Effects of reduced muscle glycogen concentration on force, Ca^{2+} release and contractile protein function in intact mouse skeletal muscle

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1. The purpose of this study was to examine the effects of reduced glycogen concentration on force, Ca^{2+} release and myofibrillar protein function during fatigue in skeletal muscle. Force and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured in single mammalian skeletal muscle fibres during fatigue and recovery. Glycogen was measured in bundles of 20–40 fibres from the same muscle under the same conditions.

2. Fatigue was induced by repeated maximum tetani until force was reduced to 30% of initial. This was associated with a reduction in muscle glycogen to 27 ± 6% of control values. In fibres allowed to recover for 60 min in the presence of 5·5 mM glucose ($n = 6$), tetanic (100 Hz) force recovered fully but tetanic $[Ca^{2+}]_i$ remained at 82 ± 8% of initial values. This prolonged depression in Ca^{2+} release was not associated with decreased muscle glycogen since glycogen had recovered to pre-fatigue levels (157 ± 42%).

3. To examine the responses under conditions of reduced muscle glycogen concentration, fibres recovered from fatigue for 60 min in the absence of glucose ($n = 6$). After glucose-free recovery, the decreases in tetanic force and $[Ca^{2+}]_i$ were only partially reversed (to 64 ± 8% and 57 ± 7% of initial values, respectively). These alterations were associated with a sustained reduction in muscle glycogen concentration (27 ± 4% of initial values).

4. In another set of fibres, fatigue was followed by 50 Hz intermittent stimulation for 22·6 ± 4 min. With this protocol, tetanic force and $[Ca^{2+}]_i$ partially recovered to 76 ± 9% and 55 ± 6% of initial levels, respectively. These changes were associated with a recovery of muscle glycogen (to 85 ± 10%).

5. During fatigue, Ca^{2+} sensitivity and maximum Ca^{2+}-activated force (F_{max}) were depressed but these alterations were fully reversed when muscle glycogen recovered. When glycogen did not recover, Ca^{2+} sensitivity remained depressed but F_{max} partially recovered. The altered myofibrillar protein function is probably due to alterations in inorganic phosphate levels or other metabolites associated with reduced levels of muscle glycogen.

6. These data indicate that the reductions in force, Ca^{2+} release and contractile protein inhibition observed during fatigue are closely associated with reduced muscle glycogen concentration. These findings also suggest that the changes in Ca^{2+} release associated with fatigue and recovery have two components – one which is glycogen dependent and another which is independent of glycogen but depends on previous activity.

During repeated tetanic contractions of skeletal muscle there is a reduction in force output. This decline in function, referred to as muscle fatigue, has a complex aetiology which can involve various metabolic and ionic factors (see Fitts, 1994; Allen, Lännergren & Westerblad, 1995) for recent reviews). Metabolic impairment plays a major role in muscle fatigue with the extent of impairment dependent on the balance between rates of ATP utilization (intensity and duration of contractile activity) and rates of ATP supply (metabolic pathway, substrate supply and muscle fibre type). During repeated contractions at high intensities (i.e. 100% maximum force for ~3 min), ATP is resynthesized predominantly by PCr degradation and anaerobic glycolysis (Spriet, 1992). Under these conditions there is an accumulation of

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inorganic phosphate (P_i), H^+ and lactate (Vøllestad, Sejersted, Bahr, Woods & Bigland-Ritchie, 1988; Nagesser, van der Laarse & Elzinga, 1992) and fatigue is thought to result from some effect of these metabolites either on sarcoplasmic reticulum (SR) Ca^{2+} release (Westerblad & Allen, 1991; Fryer, Owen, Lamb & Stephenson, 1995) or on the contractile proteins (Cooke, Franks, Luciano & Pate, 1988; Godt & Nosek, 1989). During repeated contractions at moderate intensities (i.e. 65–80% maximum, > 30 min), oxidation of glycogen and triglyceride stores will provide most of the energy for ATP resynthesis (Spriet, Heigenhauser & Jones, 1986). Under these conditions there is little accumulation of H^+ throughout most of the exercise although PCr and glycogen depletion and P_i and lactate accumulation are evident at the point of exhaustion (Vøllestad *et al.* 1988). Under conditions of prolonged moderate-intensity exercise, the cellular mechanisms underlying the reduction in force are not well understood.

The importance of glycogen to muscle function was first shown by Bergström and co-workers (Bergström, Hermansen, Hultman & Saltin, 1967) who observed a direct correlation between muscle glycogen concentration and time to fatigue. This correlation has been verified by several others (Ahlborg, Bergström, Ekelund & Hultman, 1967; Galbo, Holst & Christensen, 1979) and it is generally accepted that during moderate intensity exercise, muscle glycogen is an obligatory substrate and that its depletion induces muscle fatigue. While the mechanism by which glycogen depletion disrupts cellular function is not known, its tight association with the SR (Entman, Keslensky, Chu & van Winkle, 1980; Fridén, Seger & Eklblom, 1989) suggests that glycogen depletion may lead to alterations in excitation–contraction coupling (E–C coupling). Studies using isolated SR vesicles prepared from muscles fatigued by prolonged exercise to exhaustion have associated reductions in SR Ca^{2+}-ATPase activity (Belcastro, Rossiter, Low & Sopper, 1981; Byrd, Bode & Klug, 1989) and reductions in SR Ca^{2+} release (Favero, Pessag & Klug, 1993) with fatigue. Byrd *et al.* (1989) noted that the extent of SR Ca^{2+}-ATPase depression correlated with the extent of muscle glycogen utilization. However, a reduction in SR Ca^{2+} pump activity increases tetanic intracellular free calcium concentration ($[Ca^{2+}]_i$) and force, and is not the cause of the reduction in force during fatigue (Westerblad & Allen, 1994). In contrast, SR Ca^{2+} release failure would contribute to the loss in force observed during fatigue (Allen, Lee & Westerblad, 1989; Westerblad & Allen, 1991).

The purpose of this study was to examine the effects of reduced glycogen concentration on force and Ca^{2+} release during fatigue in single skeletal muscle fibres. We also examined the changes in Ca^{2+} sensitivity and maximum Ca^{2+}-activated force to assess the effects of altered muscle glycogen content on contractile protein function. Our findings indicate that reduced levels of muscle glycogen result in an earlier reduction in $[Ca^{2+}]_i$ and force and a shorter time to fatigue. This reduction in force can be explained by decreased Ca^{2+} release from the SR and reduced Ca^{2+} sensitivity and maximum force-generating capacity of the myofibrillar proteins. Our data also indicate that while impaired myofibrillar protein function is always associated with changes in muscle glycogen content, Ca^{2+} release failure is due to both glycogen-dependent and glycogen-independent mechanisms.

METHODS

Mice were killed by cervical dislocation and single fibres or fibre bundles were dissected from the flexor brevis muscle. Methods for fibre dissection and mounting were similar to those previously used in this laboratory (Westerblad & Allen, 1991). The mean diameter of single fibres was 40 ± 3·5 μm and the fibre bundles consisted of 20–40 fibres. Both single fibres and fibre bundles were clamped at each tendon by platinum foil micro-clips, which were attached to a force transducer at one end and to an adjustable holder at the other end. The adjustable holder allowed the single fibres and bundles to be set to the length which gave maximum tetanic tension. All preparations were superfused at 22 °C with a solution containing (mM): NaCl, 121; KCl, 5·0; $CaCl_2$, 1·8; $MgCl_2$, 0·5; NaH_2PO_4, 0·4; $NaHCO_3$, 24; and glucose, 5·5. The solution was bubbled with 95% O_2 and 5% CO_2 to give a pH of 7·3. Approximately 0·2% fetal calf serum was added.

Single fibres were used for studies measuring force and Ca^{2+} during fatigue, and fibre bundles were used for studies measuring changes in glycogen concentration. Both the single fibres and fibre bundles were stimulated with platinum electrodes using pulses of 0·5 ms duration at an intensity of approximately 1·2 × threshold. All tetanic contractions were 0·35 s in duration. Force produced during unfused tetani was assessed by the mean output over the final 100 ms.

Recording of fluorescence signals

$[Ca^{2+}]_i$ was measured with the fluorescent dye indo-1 which was micro-injected into the fibres. The experimental set-up was similar to that described by Lee, Westerblad & Allen (1991) but with a single illumination wavelength of 360 nm and dual emission wavelengths at 400 and 510 nm. Emitted light was measured by two photomultiplier tubes and passed on to an analogue divide circuit which allowed continuous monitoring of the 400/510 ratio signal. The background fluorescence was small, and was subtracted from all measurements. The indo-1 ratio was converted to $[Ca^{2+}]_i$ using the *in vivo* calibration previously described (Westerblad & Allen, 1993). The tetanic $[Ca^{2+}]_i$ was defined by the mean output over the last 100 ms.

Experimental protocol

The aim of the study was to measure muscle glycogen during fatigue and to assess the extent to which reduced glycogen concentration may be responsible for the reductions in force and $[Ca^{2+}]_i$ that have previously been observed (Westerblad & Allen, 1991; Westerblad, Duty & Allen, 1993). We used a protocol shown to fatigue single mouse muscle fibres (Westerblad & Allen, 1991) and extended this regime to include several repeated fatigue bouts. Various conditions were designed to manipulate muscle glycogen levels between fatigue bouts. The standard fatigue protocol consisted of repeated maximal tetani continued until muscle force

was reduced to less than 30%. Fibres were stimulated at 100 Hz for 0·35 s with tetani repeated at an interval of 4 s for the first 2 min, and the interval reduced every 2 min to 3 s, 2·5 s, 2·0 s, and so on. Under control conditions (Con), this protocol was followed by 60 min of recovery during which the fibres were continuously perfused with a standard solution containing 5·5 mM glucose. After 60 min, fibres were fatigued a second time using the same 100 Hz stimulation protocol described above. The repeated fatigue bouts will be referred to as fatigue 1 and fatigue 2 for the first and second fatigue bout, respectively. Fatigue resistance, defined as the time required to reach 30% of initial force, was compared between fatigue 1 and fatigue 2. The same absolute level of force (i.e. 30% of initial force during fatigue 1) was used to assess fatigue resistance for each fatigue run. In some fibres a third and fourth fatigue bout were also given, each separated by 60 min of recovery.

To examine the effects of reduced muscle glycogen concentration on the changes in force and $[Ca^{2+}]_i$ during fatigue, two other conditions were examined. These conditions were designed to manipulate the glycogen content of the single fibres at the start of the second fatigue bout. In the first condition, a solution with no glucose was used to perfuse the fibres during the 60 min recovery period between fatigue 1 and fatigue 2. It was expected that this intervention would prevent resynthesis of any glycogen used during the first fatigue bout. In some fibres ($n = 3$), glucose was removed during, as well as between, both fatigue bouts (−Gluc). In the second condition, fibres were stimulated (Stim) using a pattern of 50 Hz intermittent tetani between fatigue 1 and fatigue 2 in a solution containing glucose. The Stim protocol consisted of 50 Hz tetani repeated at a rate of 1 every 10 s for 2 min, then 1 every 9 s for 2 min and so on, with the tetanus rate increased every 2 min until 50 Hz force reached 30% of its initial value. On average, fibres were stimulated 22·6 ± 4 min. There was a brief period of recovery (9 min) between fatigue 1 and the 50 Hz tetani and again between the 50 Hz tetani and fatigue 2, during which force and $[Ca^{2+}]_i$ were assessed at a range of frequencies (see details below). This protocol was designed to gradually reduce muscle glycogen stores below the levels obtained at the end of fatigue 1 as a way of examining changes during fatigue at moderate work intensities. In preliminary studies we also attempted this stimulation protocol in a glucose-free solution but no fibres survived the protocol.

Contractile protein function assessment

In order to determine the Ca^{2+} sensitivity and maximum Ca^{2+}-activated force, we used a protocol previously described (Westerblad & Allen, 1991). Briefly, before each fatigue bout, fibres were activated at a range of frequencies (30, 50, 70, 100 Hz and 100 Hz in the presence of 5 mM caffeine) at 1 min intervals. Force and $[Ca^{2+}]_i$ at each frequency were used to construct force–Ca^{2+} curves and to determine the resting Ca^{2+} sensitivity and maximum Ca^{2+}-activated force. Force–Ca^{2+} curves were fitted using a Hill equation:

$$F = F_{max}[Ca^{2+}]_i^n/(Ca_{50}^n + [Ca^{2+}]_i^n),$$

where F is force, F_{max} is the force at saturating $[Ca^{2+}]_i$, n is a constant describing the steepness of the relation and Ca_{50} represents the $[Ca^{2+}]_i$ required for 50% of maximum force. To assess the changes in Ca^{2+} sensitivity during fatigue, force and $[Ca^{2+}]_i$ from the final phase of fatigue, when both are declining, were used (see Westerblad & Allen, 1991). Force and $[Ca^{2+}]_i$ from a 100 Hz tetanus in the presence of caffeine, representing maximum Ca^{2+}-activated force, were measured at the end of the fatigue protocol and added to the force–Ca^{2+} plot which could then be fitted by a Hill equation as described above. Values of F_{max} and Ca_{50} before and during fatigue were compared in the control (Con), no glucose (−Gluc) and 50 Hz-stimulated (Stim) groups to assess the effects of glycogen depletion on Ca^{2+} sensitivity and maximum Ca^{2+}-activated force.

Muscle glycogen determination

To quantify the changes in muscle glycogen during fatigue and recovery, these experiments were repeated in small bundles of approximately 20–40 fibres. Separate experiments were required to assess the muscle glycogen levels at the end of fatigue 1 ($n = 4$) and after recovery for control ($n = 5$), no glucose ($n = 4$) and 50 Hz stimulation ($n = 4$) conditions. For each of these samples a control bundle from the same muscle was also perfused with glucose, but not stimulated, for the same duration that the fatigued bundles were perfused. The level of glycogen in these bundles represents the pre-fatigue level of muscle glycogen. Values for stimulated muscle are expressed as a percentage of control values relative to the unstimulated bundles. Stimulated and unstimulated muscles were quick-frozen in liquid nitrogen and stored at −80 °C until analysed. Glycogen was assessed in the fibre bundles using a fluorometric method (Lowry & Passonneau, 1972). Samples were first hydrolysed in 2 N HCl at 100 °C for 2 h and then glucose concentration measured in the extract. Total protein of the extract was determined using a bicinchoninic acid (BCA) method with serum albumin as a standard. In a subset of larger samples from the same muscle, weight and protein content were measured and the protein yield determined (136 ± 14 mg g^{-1}). This value was used to express glycogen concentrations in micromoles of glucosyl units per gram wet weight of tissue (μmol (g wet wt)$^{-1}$).

Statistical analyses

All values are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) for repeated measures was used to test for statistical differences within each group (pre- *vs.* post-fatigue). One-way random block ANOVAs were used to test for differences between groups (Con *vs.* −Gluc *vs.* Stim). Significance was accepted at $P < 0·05$.

RESULTS

Force output during repeated fatigue bouts

Using our standard fatigue protocol, force was reduced to 30% of initial values in 222 ± 44 s. Under control conditions where the fibres recovered between fatigue bouts for 60 min in the presence of glucose, fatigue resistance was not significantly different in the second fatigue bout (mean time to fatigue, 243 ± 54 s; $n = 6$). Figure 1 shows the force recorded from a representative fibre in which four successive fatigue bouts were given. With glucose present during recovery, there was minimal change in fatigue resistance even after four fatigue bouts.

To assess the effects of reduced muscle glycogen concentration on fatigue resistance, time to fatigue was measured after fatigued fibres recovered for 60 min in the absence of glucose (−Gluc rec). Under these conditions, fatigue resistance was significantly reduced (from 273 ± 50 to 38 ± 17 s; $n = 3$). Figure 2 shows the force recorded from a representative fibre that recovered without glucose between fatigue 1 and fatigue 2. The time to fatigue in the second fatigue run was abbreviated, with force declining rapidly immediately after

the first tetanus. This reduction in fatigue resistance was readily reversed when the fibre was allowed to recover for 60 min in the presence of glucose after fatigue 2. Note the similar time to fatigue during fatigue 3 and fatigue 1. The improved fatigue resistance with an additional period of recovery with glucose indicates that the reductions in fatigue resistance were not due to permanent impairment of the fibre but were related to the changes in substrate supply.

We were also interested in whether fatigue resistance would be altered by the presence or absence of glucose during fatigue. In fibres where glucose was removed during fatigue 1 and fatigue 2 as well as during recovery (–Gluc), fatigue resistance was not significantly reduced during fatigue 1 (125 ± 28 s, $P = 0{\cdot}113$) but was reduced during fatigue 2 compared with fatigue 1 (30 ± 2 s). Since the relative reduction in fatigue resistance during fatigue 2 was similar for –Gluc rec (16%) and –Gluc (18%) and subsequent analyses revealed that changes in force and $[Ca^{2+}]_i$ were also similar for these conditions, data for these two groups were pooled (–Gluc; $n = 6$). In four other fibres that were fatigued once with glucose and once without glucose (glucose present during recovery; data not shown), fatigue resistance was not altered (202 ± 16 *vs.* 192 ± 27 s). This indicates that glucose removal during recovery was more important

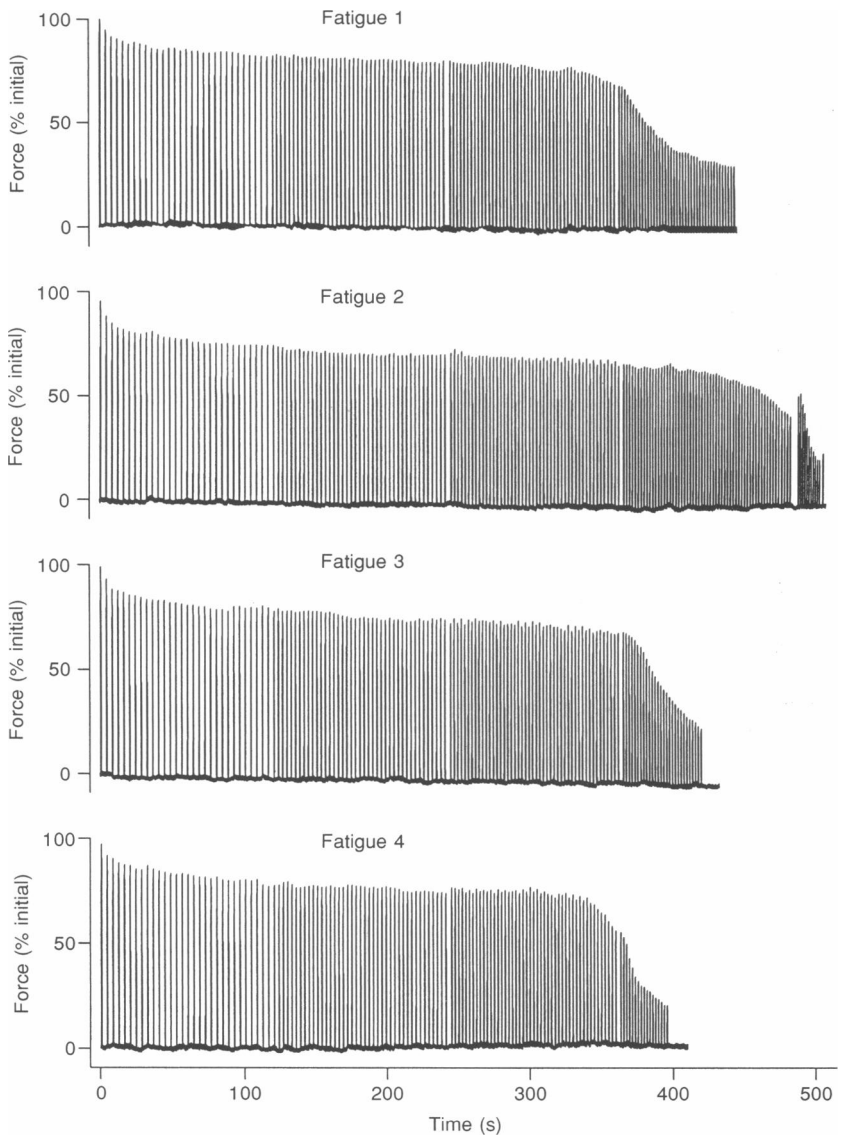

Figure 1. Force output from a single fibre under control conditions

The fibre was fatigued by intermittent tetani (0·35 s every 4 s) with the duty cycle increased every 2 min until force output reached 30% of the initial levels. Between each fatigue bout the fibre recovered unstimulated for 60 min in the presence of 5·5 mM glucose. Fatigue resistance, determined by the time to reach 30% initial force, was not altered even after 4 fatigue bouts. Values represent the percentage of initial 100 Hz force, with 100% representing the force at the beginning of fatigue 1.

than glucose removal during fatigue in altering the fatigue resistance of the fibres. It also indicates that in these predominantly type II fibres (Allen, Duty & Westerblad, 1993), glucose uptake from the perfusate does not contribute much to the energy required for contractions and suggests that intracellular stores of PCr and glycogen provide most of the fuel for ATP resynthesis.

Fatigue resistance was also assessed in a group of fibres where a period of 50 Hz tetani was given after the first fatigue bout. Under these conditions, time to fatigue was reduced from 333 ± 78 to 187 ± 77 s ($n = 7$). Figure 3 shows the force output of a fibre where 50 Hz stimulation in the presence of glucose was given between the first and second fatigue bout. In this fibre, fatigue resistance was reduced after 50 Hz stimulation (fatigue 2) but increased again after 60 min of recovery with glucose (fatigue 3). The reduced fatigue resistance following low-frequency stimulation is therefore not due to permanent damage of the fibre but probably related to metabolic or ionic alterations at the start of fatigue 2.

Force recovery between fatigue runs

The changes in force in response to fatigue under the different conditions is shown in Fig. 4. The mean 100 Hz tetanic force produced by the single fibres was 44·1 ± 1·7 mg or 350 ± 13 kPa when normalized for cross-sectional area, similar to previously reported values (Westerblad & Allen, 1991). Under control conditions, force at the start of fatigue 2 was 100 ± 2% of initial values, indicating that force had been fully restored during 60 min of recovery with glucose. Under conditions where no glucose was present during recovery, force at the beginning of fatigue 2 was reduced to 64 ± 8% of force at the beginning of fatigue 1.

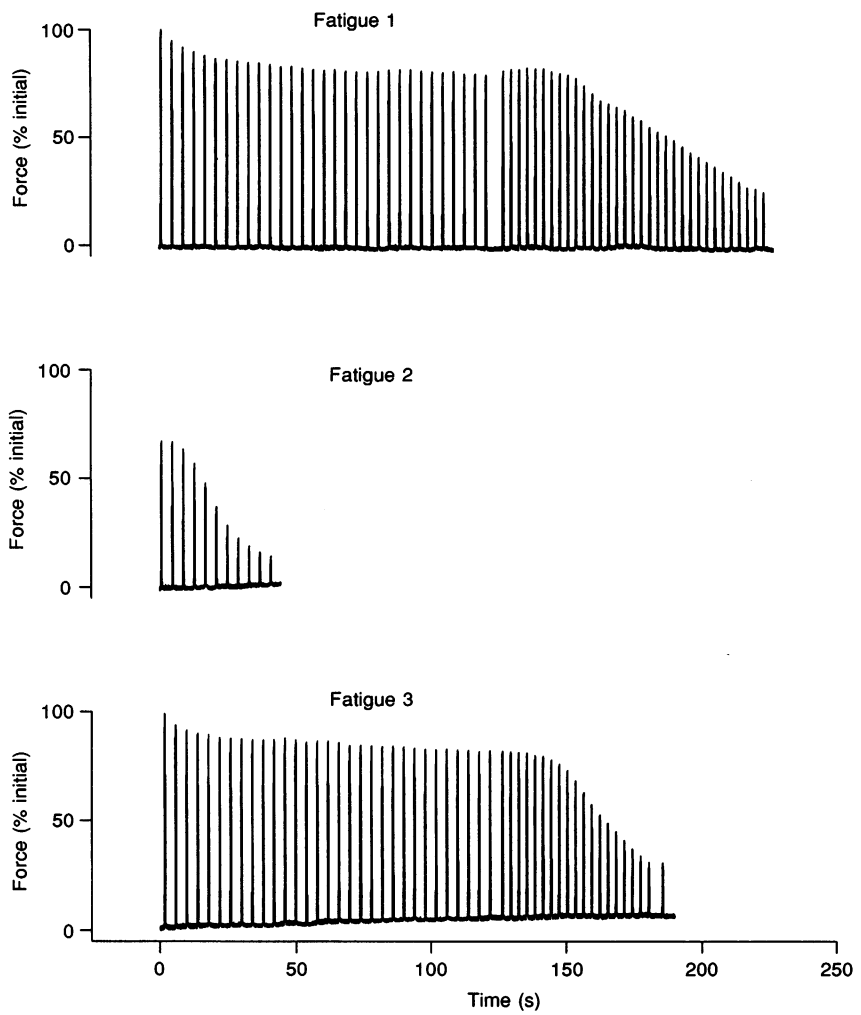

Figure 2. Force output from a single fibre after recovery without glucose between fatigue 1 and fatigue 2

The fibre was fatigued using the same protocol described for Fig. 1. Between fatigue 1 and fatigue 2, the fibre recovered unstimulated for 60 min in the absence of glucose; 5·5 mM glucose was present during each of the fatigue bouts. The period of glucose-free recovery resulted in a reduction in fatigue resistance during fatigue 2 which could be reversed following subsequent recovery with glucose (fatigue 3). Tetanic force was also reduced at the beginning of fatigue 2 and recovered before fatigue 3.

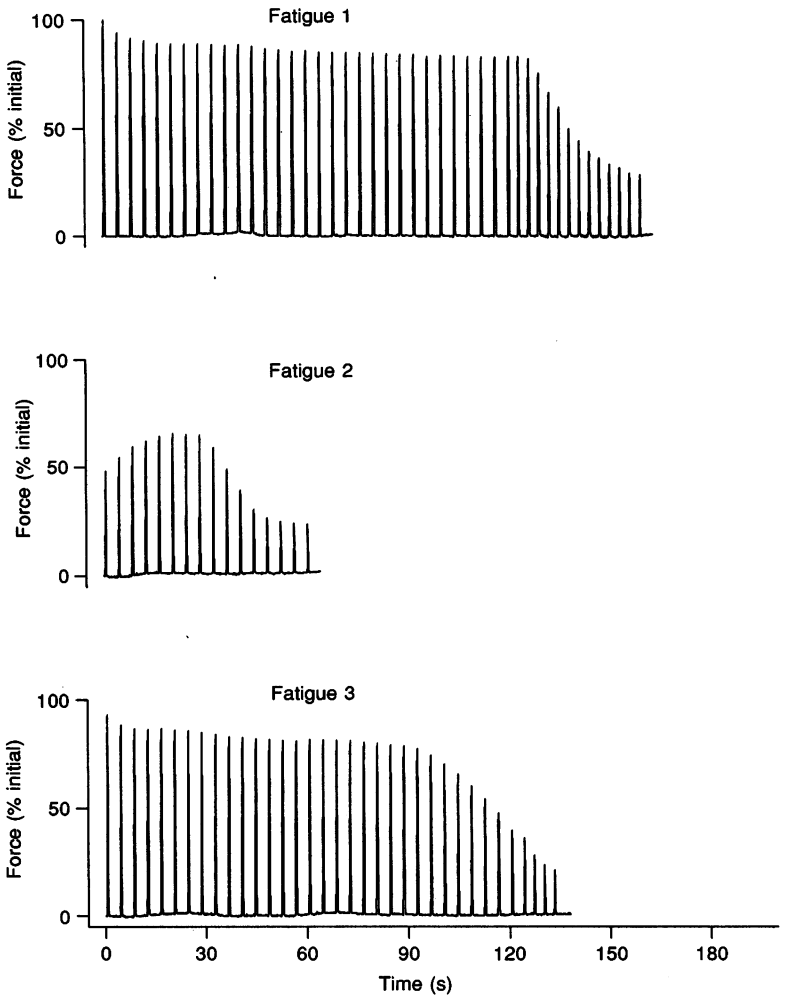

Figure 3. Force output from a single fibre after 50 Hz stimulation between fatigue 1 and fatigue 2

The fibre was fatigued using the protocol described for Fig. 1. Between fatigue 1 and fatigue 2, the fibre was stimulated with intermittent 50 Hz tetani at a rate of 1 every 10 s for 2 min, then 1 every 9 s for 2 min, etc. with the train rate increased every 2 min until force reached 30% of the initial 50 Hz force. For this fibre, 50 Hz stimulation was stopped after 11 min. Fatigue 2 followed after a brief rest (9 min) during which force and $[Ca^{2+}]_i$ were measured at various frequencies. After fatigue 2, the fibre recovered unstimulated for 60 min. Glucose (5·5 mM) was present in the perfusing solution throughout the entire experiment. The period of 50 Hz stimulation resulted in a decrease in fatigue resistance during fatigue 2 which was reversed with unstimulated recovery before fatigue 3. Tetanic force was reduced at the start of fatigue 2 but also recovered by the start of fatigue 3.

In fibres stimulated at 50 Hz between the first and second fatigue bouts there was also a significant depression in tetanic force at the start of fatigue 2 (76 ± 9% of initial levels). Comparing the differences between groups, 100 Hz force at the beginning of fatigue 2 was significantly reduced in the −Gluc and Stim groups compared with the Con group.

Intracellular Ca^{2+} recovery between fatigue runs

In order to determine whether reductions in Ca^{2+} release contributed to the reductions in force under the different conditions, changes in $[Ca^{2+}]_i$ were also assessed. Examples of the alterations in $[Ca^{2+}]_i$ associated with the changes in force during fatigue 1 and fatigue 2 under control and

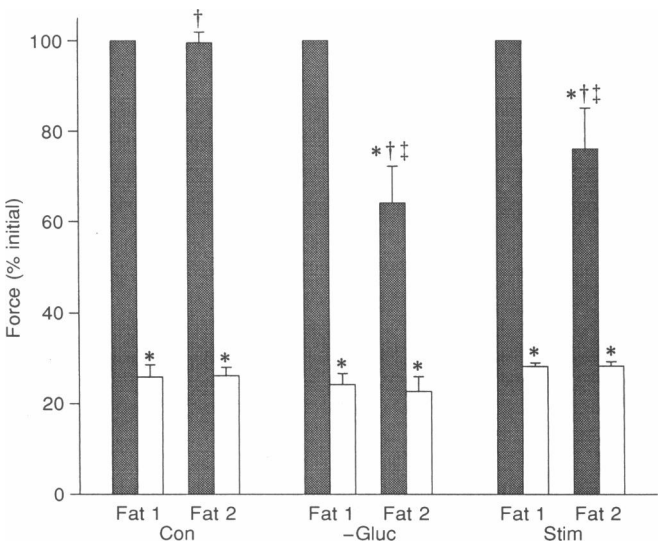

Figure 4. Mean changes in force during fatigue 1 and fatigue 2 with different recovery conditions

Force at the beginning of fatigue 1 (Fat 1) and fatigue 2 (Fat 2) (▨) and at the end of each fatigue bout (□) is shown for the different conditions: control (Con) where fibres recovered in the presence of 5·5 mM glucose; glycogen depleted (−Gluc) where fibres recovered without glucose; and stimulation (Stim) where fibres received 50 Hz tetani intermittently over 23 ± 4 min in the presence of 5·5 mM glucose. Force for each fibre was expressed as a percentage of its initial 100 Hz force. * $P < 0.05$ *vs.* first tetanus for fatigue 1. † $P < 0.05$ *vs.* last tetanus of fatigue 1. ‡ $P < 0.05$ *vs.* same time point for Con.

glycogen-reduced conditions are shown in Figs 5 and 6, respectively. Figure 5 shows that under control conditions, fatigue 1 resulted in a decrease in $[Ca^{2+}]_i$ from 1144 to 590 nM when force fell to 30% of initial (Fig. 5*a vs. b*). Following 60 min of recovery with glucose, $[Ca^{2+}]_i$ had recovered to 791 nM when force had recovered to its starting level (Fig. 5*c*). During fatigue 2, force declined with a similar time course to that in fatigue 1 and $[Ca^{2+}]_i$ fell to 635 nM, a level not different from that at the end of fatigue 1 (Fig. 5*b vs. d*). These results can be compared with Fig. 6, which shows the diminished recovery of $[Ca^{2+}]_i$ and force when glucose was not present during recovery. Fatigue 1 resulted in a reduction of $[Ca^{2+}]_i$ from 944 to 519 nM when force was reduced to 30% (Fig. 6*a vs. b*). After 60 min of recovery without glucose, $[Ca^{2+}]_i$ had recovered to only 626 nM and force to only 82% of initial (Fig. 6*c*). During fatigue 2, $[Ca^{2+}]_i$ fell to 460 nM when force was reduced to 30%, which was similar to $[Ca^{2+}]_i$ at the end of fatigue 1.

A summary of the changes in $[Ca^{2+}]_i$ in response to fatigue under the different conditions is shown in Fig. 7. The mean tetanic $[Ca^{2+}]_i$ at the start of fatigue 1 was 887 ± 57 nM, which was reduced to 47 ± 3% at the end of fatigue 1. Under control conditions, the recovery of $[Ca^{2+}]_i$ to 82 ± 8% of initial levels by the start of fatigue 2 is consistent with previous observations of prolonged Ca^{2+} release failure following this type of fatigue protocol (Westerblad *et al.* 1993). At the end of fatigue 2, $[Ca^{2+}]_i$ was again reduced to 48 ± 5% of the initial level, a level not different from that at the end of fatigue 1. In fibres where glucose was removed during recovery, $[Ca^{2+}]_i$ during the first tetanus of fatigue 2 was 57 ± 7% of the initial level. Intracellular $[Ca^{2+}]$ at the end of fatigue 2 was reduced to 38 ± 4%, a level not different from that at the end of fatigue 1. In fibres stimulated at 50 Hz between fatigue 1 and fatigue 2, the changes in $[Ca^{2+}]_i$ followed a similar pattern. In seven fibres, the mean $[Ca^{2+}]_i$ during the first tetanus of fatigue 2 was 55 ± 6% of initial. At the end of fatigue 2, $[Ca^{2+}]_i$ was reduced to 41 ± 3% of initial and was not different from $[Ca^{2+}]_i$ at the end of fatigue 1. Comparing the differences between groups, $[Ca^{2+}]_i$ at the start of fatigue 1 was similar

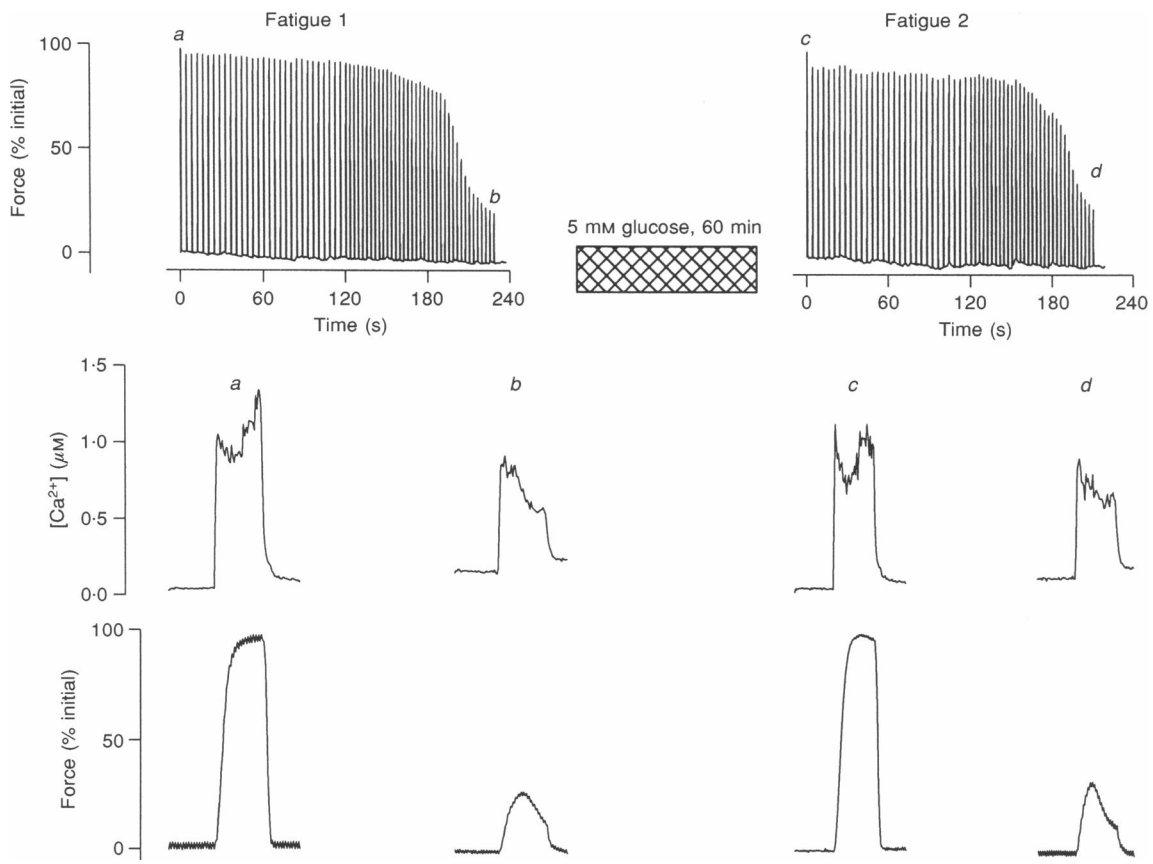

Figure 5. Data traces representative of the changes in force, $[Ca^{2+}]_i$ and time to fatigue for fatigue 1 and fatigue 2 under control conditions

Tetanic (100 Hz) force and $[Ca^{2+}]_i$ were reduced at the end of fatigue 1 (*a vs. b*). After 60 min recovery in the presence of 5·5 mM glucose, tetanic force had fully recovered but $[Ca^{2+}]_i$ remained depressed (*b vs. c*). During fatigue 2, tetanic force and $[Ca^{2+}]_i$ were reduced (*c vs. d*) over a similar time course as fatigue 1. Intracellular $[Ca^{2+}]$ reached the same level at the end of both fatigue bouts (*b vs. d*) despite starting at lower levels at the beginning of fatigue 2.

for all conditions. However, the $[Ca^{2+}]_i$ at the start of fatigue 2 was significantly reduced in the −Gluc and Stim groups compared with the Con group.

Ca^{2+} sensitivity changes between fatigue runs

The reductions in force observed during fatigue can be attributed not only to reductions in $[Ca^{2+}]_i$ but also to reductions in Ca^{2+} sensitivity of the myofibrils and maximum Ca^{2+}-activated force. Table 1 shows the changes in Ca^{2+} sensitivity (Ca_{50}) and in maximum Ca^{2+}-activated force (F_{max}) obtained from Hill plots of the force–Ca^{2+} relationships under the different conditions. Fatigue 1 resulted in an increase in Ca_{50} and a decrease in F_{max} in all three groups, which is consistent with previous reports (Westerblad & Allen, 1991). After 60 min of recovery with glucose, Ca_{50} and F_{max} had returned to pre-fatigue levels but during fatigue 2 were again altered to levels not different from fatigue 1. In fibres that recovered without glucose, alterations in Ca_{50} and F_{max} were not completely reversed by 60 min, indicating that recovery of contractile protein inhibition was incomplete. Between fatigue 1 and fatigue 2 there was no recovery of Ca_{50}, but there was a partial recovery of F_{max}. During the abbreviated second fatigue bout there was no further reduction in Ca^{2+} sensitivity but F_{max} decreased to levels similar to fatigue 1. In fibres stimulated at 50 Hz after fatigue 1, Ca_{50} and F_{max} had returned to pre-fatigue levels by the start of fatigue 2. The reversible decrease in Ca_{50} and F_{max} indicates that the alterations in Ca^{2+} sensitivity and maximum Ca^{2+}-activated force induced during fatigue from 100 Hz tetani had recovered during the period of 50 Hz stimulation. During fatigue 2, Ca_{50} and F_{max} decreased again to levels similar to fatigue 1. Comparing the differences between groups, Ca_{50} and F_{max} were altered to a similar extent at all times except at the beginning of fatigue 2, when Ca_{50} was increased and F_{max} decreased in the −Gluc compared with the Con and Stim groups.

Muscle glycogen concentrations

Muscle glycogen concentration was measured in fibre bundles that were fatigued using protocols identical to those described for the single-fibre studies. In the bundles, the

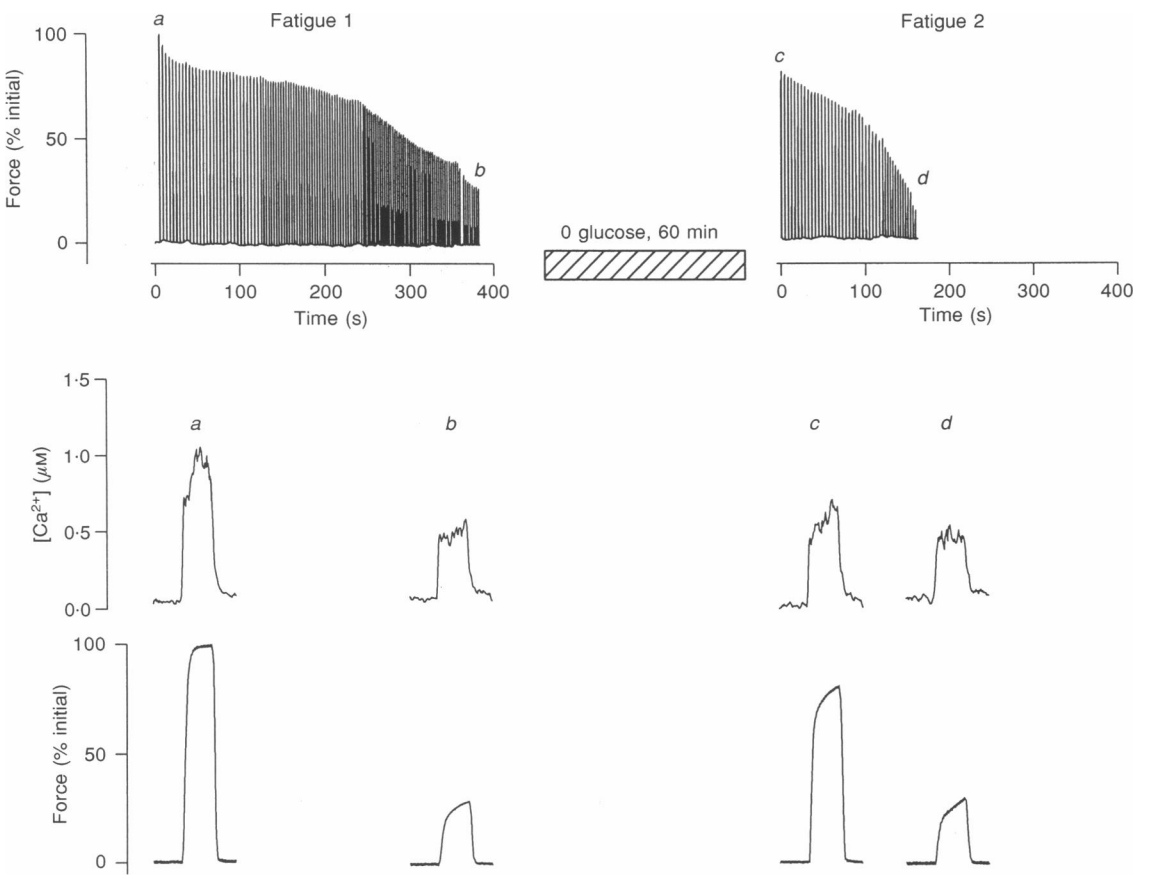

Figure 6. Data traces showing the changes in force, $[Ca^{2+}]_i$ and time to fatigue for fatigue 1 and fatigue 2 for a fibre that recovered without glucose

Tetanic (100 Hz) force and $[Ca^{2+}]_i$ were reduced at the end of fatigue 1 (*a vs. b*). After 60 min recovery without glucose, tetanic force only partially recovered and $[Ca^{2+}]_i$ showed only minor improvement (*b vs. c*). During fatigue 2, tetanic force and $[Ca^{2+}]_i$ were reduced (*c vs. d*) more rapidly compared with fatigue 1. Intracellular $[Ca^{2+}]$ reached the same level at the end of both fatigue bouts (*b vs. d*) despite starting at lower levels and declining more rapidly during fatigue 2.

Table 1. Force–Ca^{2+} relationships during repetitive fatigue bouts under control and glycogen-depleted conditions

	Ca_{50} (nM)	F_{max}
Con		
Pre-fatigue 1	355 ± 37	100%
Fatigue 1	456 ± 49*	84·9 ± 2·0%*
Pre-fatigue 2	367 ± 45†	100·7 ± 2·0%†
Fatigue 2	449 ± 47*‡	82·4 ± 1·4%*†‡
−Gluc		
Pre-fatigue 1	348 ± 30	100%
Fatigue 1	425 ± 47*	80·3 ± 4·0%*
Pre-fatigue 2	445 ± 33*	89·2 ± 2·6%*†
Fatigue 2	455 ± 38*	76·4 ± 3·6%*‡
Stim		
Pre-fatigue 1	344 ± 36	100%
Fatigue 1	475 ± 25*	87·3 ± 2·7%*
Pre-fatigue 2	356 ± 42†	96·9 ± 1·5%†
Fatigue 2	437 ± 39*	84·7 ± 1·9%*‡

* $P < 0·05$ *vs.* pre-fatigue 1. † $P < 0·05$ *vs.* fatigue 1. ‡ $P < 0·05$ *vs.* pre-fatigue 2.

mean force was 1077 ± 58 mg and the mean time to fatigue for fatigue 1 was 530 ± 52 s. Although the time to fatigue was substantially longer for the fibre bundles, suggesting that the single fibres we dissect are generally the larger and more easily fatigable fibre types, the changes in fatigue resistance in the various protocols were similar to those observed in the single fibre studies. For fatigue 2, fatigue resistance was not different when muscles recovered with glucose (94 ± 4% of initial values) but was reduced following recovery without glucose (55 ± 11% of initial values). Muscle glycogen concentration in non-stimulated bundles averaged 26·0 ± 3 μmol (g wet wt)$^{-1}$. Figure 8 shows the changes in glycogen concentration after fatigue 1 and after recovery in the three groups. After fatigue 1 there was a significant reduction in muscle glycogen to 27 ± 6% of non-stimulated muscle levels. When the bundles were allowed to recover for 60 min in the presence of glucose, glycogen returned to levels not different from those of unstimulated muscle (157 ± 42%), indicating that glycogen resynthesis was complete within this time frame. The recovery of glycogen between fatigue 1 and fatigue 2 is consistent with the observation that fatigue resistance was similar for the two fatigue runs under control conditions. When glucose was not present during the 60 min of recovery, muscle glycogen remained significantly lower than non-stimulated muscle (27 ± 4%). This indicates that recovery without glucose prevented glycogen resynthesis and is consistent with the reduction in fatigue resistance for fatigue 2 (17% of fatigue 1). When fibres were stimulated at 50 Hz between fatigue 1 and fatigue 2, muscle glycogen was 85 ± 10% of unstimulated muscle, a level which was not different from control. Although we expected this protocol to further reduce glycogen concentration, glycogen repletion was probably stimulated by the recruitment of glucose transporters during the 50 Hz stimulation (Douen *et al.* 1990). The recovery of muscle glycogen is consistent with the recovery of Ca^{2+} sensitivity and F_{max} during 50 Hz stimulation but does not explain the reduced fatigue resistance during fatigue 2.

DISCUSSION

In this study we have investigated the role that reduced muscle glycogen concentration plays in the failure of Ca^{2+} release and altered myofibrillar protein function observed during muscle fatigue. Under conditions where muscle glycogen concentration was reduced to 27%, the reduction in $[Ca^{2+}]_i$ and force was accelerated, suggesting that glycogen availability is important for normal Ca^{2+} release. When fibres were not stimulated during recovery, restoration of force and Ca^{2+} release were associated with the resynthesis of muscle glycogen. Stimulation during recovery did not

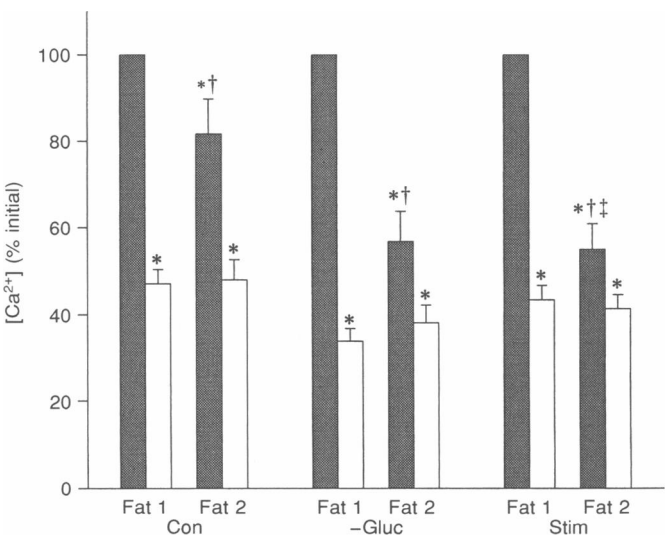

Figure 7. Mean changes in intracellular free $[Ca^{2+}]$ during fatigue 1 and fatigue 2 with different recovery conditions

$[Ca^{2+}]_i$ at the beginning of fatigue 1 (Fat 1) and fatigue 2 (Fat 2) (▩) and at the end of each fatigue bout (□) are shown for the different conditions: control (Con) where fibres recovered in the presence of 5·5 mM glucose; glycogen depleted (−Gluc) where fibres recovered without glucose; and stimulation (Stim) where fibres received 50 Hz tetani intermittently over 23 ± 4 min in the presence of 5·5 mM glucose. $[Ca^{2+}]_i$ for each fibre was expressed as a percentage of its initial 100 Hz $[Ca^{2+}]_i$. * $P < 0·05$ *vs.* first tetanus of fatigue 1. † $P < 0·05$ *vs.* last tetanus of fatigue 1. ‡ $P < 0·05$ *vs.* same time point for Con.

prevent glycogen resynthesis but did impair the recovery of force and Ca^{2+} release. We therefore postulate that changes in force and Ca^{2+} release involve both glycogen-dependent and glycogen-independent mechanisms. Alterations in Ca^{2+} sensitivity and maximum Ca^{2+}-activated force of the myofibrillar proteins were correlated with changes in muscle glycogen and are probably due to the alterations in P_i or other metabolites associated with glycogen breakdown and resynthesis.

Muscle glycogen depletion and fatigue resistance

In the present study, glycogen concentration measured in small bundles of the flexor brevis muscle of mice averaged 26 μmol (g wet wt)$^{-1}$, values within the range reported for mouse fast twitch muscle (Bonen & Homonko, 1994). Intermittent tetanic stimulation resulted in reductions in glycogen concentration to ~25%. This is consistent with studies in rat skeletal muscle where a reduction of muscle glycogen to 27% was observed with a comparable stimulation protocol, and a decrease to 40% was observed with exhaustive swimming (Lindinger, Heigenhauser & Spriet, 1987). Although we were unable to measure glycogen in single fibres, we would expect the level of glycogen utilization to be similar since the stimulation protocol was the same. Based on the reduction in time to fatigue following recovery without glucose (to 17% of initial values in single fibres *vs.* 55% in fibre bundles), glycogen in the single fibres may have been reduced to even less than the 25% estimated from the fibre bundles. In interpreting our results we have assumed that muscle glycogen levels in the single fibres were similar to those in the fibre bundles treated with the same protocol.

We have shown that fatigue resistance within a given muscle fibre, is associated with the availability of glycogen. These data are consistent with the ideas proposed by Bergström *et al.* (1967) that muscle glycogen concentration is important in determining muscle performance during prolonged exercise. We have also extended this idea by showing that both force and Ca^{2+} release are associated with glycogen concentration. At the cellular level, glycogen concentration may alter fatigue resistance by limiting the duration for which E–C coupling mechanisms can function normally before Ca^{2+} release fails and $[Ca^{2+}]_i$ and force start to fall. A surprising observation was that fatigue resistance was not impaired by the small but prolonged reduction in Ca^{2+} release induced by a single fatigue bout. This suggests that the duration of a bout of activity depends primarily on the available energy supply, in this case glycogen, and small reductions in Ca^{2+} release do not affect the endurance capacity of a fibre. However, when the prolonged reductions of Ca^{2+} release are greater than 20%, such as observed when 50 Hz stimulation followed a single fatigue bout, then the endurance of a fibre is compromised despite sufficient glycogen availability. Thus, prolonged reductions in E–C coupling do not reduce fatigue resistance until the impairments are greater than the extent observed after a single fatigue bout.

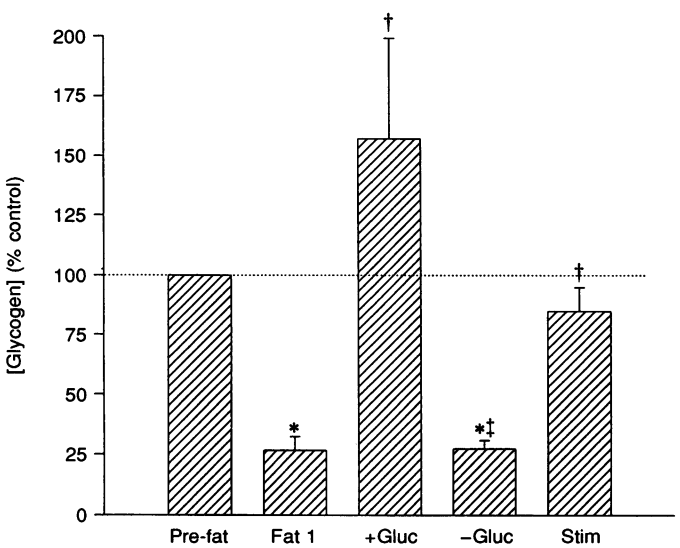

Figure 8. Mean muscle glycogen values obtained in fibre bundles after fatigue 1 and following recovery under the different conditions

Glycogen concentrations are expressed as a percentage of the values in a control bundle from the same animal that was perfused but unstimulated for 60 min in the presence of 5·5 mM glucose. In the control bundles, glycogen concentration averaged 26 ± 3 μmol (g wet wt)$^{-1}$, which is shown as the pre-fatigue glycogen level (Pre-fat). Glycogen levels are also shown for bundles after fatigue 1 (Fat 1), after recovery from fatigue 1 for 60 min with 5·5 mM glucose (+Gluc), after recovery from fatigue 1 for 60 min without glucose (−Gluc) and after intermittent 50 Hz tetani following fatigue 1 (Stim). * $P < 0.05$ *vs.* Pre-fat. † $P < 0.05$ *vs.* Fat 1. ‡ $P < 0.05$ *vs.* +Gluc.

Muscle glycogen and Ca^{2+} release failure during fatigue

During the intermittent stimulation protocol used there are several phases of fatigue, differentiated by the changes in force and $[Ca^{2+}]_i$, which help to identify different underlying mechanisms. These have previously been shown in single muscle fibres from both mouse (Lännergren & Westerblad, 1991) and frog (Nagesser *et al.* 1992) and include an early reduction in force (phase 1), a plateau phase (phase 2) and a final linear fall in force (phase 3). During phase 1, the 15% reduction in force over the first eight to ten tetani is due to an inhibition of the contractile proteins (Lännergren & Westerblad, 1991) caused by an accumulation of metabolites following rapid activation of PCr hydrolysis and anaerobic glycolysis (Nagesser *et al.* 1992). The start of phase 2 is thought to coincide with a metabolic steady state where oxidative metabolism can meet the ATP demands of the repetitive contractions (Lännergren & Westerblad, 1991). During this metabolic steady state, muscle glycogen appears to be the predominant substrate for ATP resynthesis. This is consistent with the observation that phases 1 and 2 were abbreviated or completely absent when muscle glycogen concentration was reduced (−Gluc) and that repletion of glycogen between the second and third fatigue bouts allowed this plateau phase to be re-established. In phase 3, force and $[Ca^{2+}]_i$ fall rapidly due to failure of E–C coupling mechanisms (Westerblad & Allen, 1991). When glycogen was reduced, fibres went into phase 3 immediately or much sooner, suggesting that with low levels of starting glycogen there is no metabolic steady state. The prevailing metabolic conditions under these circumstances caused an immediate failure of Ca^{2+} release and force loss. An important observation from this study is that the mechanism responsible for the decrease in force and $[Ca^{2+}]_i$ (i.e. E–C coupling failure) observed during phase 3 in fatigue is not different when muscle glycogen concentration is reduced. Instead, the time course is altered, with muscle glycogen being utilized earlier and E–C coupling failure occurring much sooner. Thus, the fall in force during phase 3 under control conditions would also appear to be due to a glycogen-dependent failure of E–C coupling, albeit much later in the fatigue protocol.

Muscle glycogen and myofibrillar protein function during fatigue

The decline in force observed during muscle fatigue has been attributed to an inhibition of the myofibrillar proteins as well as to impaired Ca^{2+} release from the SR (Fitts, 1994; Allen *et al.* 1995). Inhibition of the myofibrillar proteins is due primarily to increases in P_i and H^+ which have been shown to depress maximum Ca^{2+}-activated force (Cooke *et al.* 1988; Godt & Nosek, 1989) and Ca^{2+} sensitivity (Godt & Nosek, 1989) of skinned single fibres. It has previously been shown, however, that there are no large increases in $[H^+]$ in intact single mouse fibres with this fatigue protocol (Westerblad & Allen, 1992). Therefore the changes in contractile protein function in this preparation are presumably due to an accumulation of P_i. A novel observation in this study was that the changes in maximum Ca^{2+}-activated force and Ca^{2+} sensitivity (and therefore P_i) during a second fatigue bout were of a similar magnitude to those of the first fatigue bout. Also, the reductions in maximum force and Ca^{2+} sensitivity at the end of the fatigue bout were similar regardless of the starting levels of muscle glycogen. Thus, differences in starting levels of glycogen appear to alter the time course and not the magnitude of contractile protein inhibition and recovery. This would be expected if the starting level of muscle glycogen determines how long ATP can be supplied by glycolysis before the remaining PCr is hydrolysed and P_i concentration increased.

While there were no differences in the extent of contractile protein inhibition during fatigue with different starting glycogen levels, the degree of recovery over 60 min was dependent on glycogen availability. Under conditions where glycogen was resynthesized (Con, Stim) there was a full recovery of myofibrillar protein function. However, when glycogen did not recover (−Gluc) there was no recovery of Ca^{2+} sensitivity and only partial recovery of maximum Ca^{2+}-activated force. The partial recovery of maximum force in the absence of any change in Ca^{2+} sensitivity would not be expected if both effects are mediated only by the increases in P_i. Other metabolic changes, including increases in ADP or increases in AMP from the resynthesis of ATP via the myokinase reaction, may also contribute to changes in contractile protein function when glycogen does not recover. An increase in AMP has been shown to increase F_{max} without altering Ca_{50} (Godt & Nosek, 1989) and could therefore explain the partial recovery of maximum Ca^{2+}-activated force without any change in Ca^{2+} sensitivity during recovery without glucose. Although we cannot precisely determine the metabolites involved, it seems clear that myofibrillar protein function remains depressed when energy is not available for muscle glycogen resynthesis.

Glycogen-dependent and glycogen-independent mechanisms of Ca^{2+} release failure

Recovery of force and $[Ca^{2+}]_i$ after fatigue follows a complex time course. One component of force and $[Ca^{2+}]_i$ recovers over 15–30 min and then there is a slow-recovering component which requires more than 60 min (Westerblad *et al.* 1993). This complex recovery pattern and its alteration when glycogen resynthesis was impaired suggests that there are at least two types of mechanisms contributing to the failure of Ca^{2+} release during fatigue: glycogen-dependent and glycogen-independent mechanisms. The glycogen-dependent component of Ca^{2+} release failure would account for the partial recovery of $[Ca^{2+}]_i$ observed during the first hour after fatigue. In the present study, when fibres recovered in the presence of glucose, glycogen had recovered and $[Ca^{2+}]_i$ increased from 47 to 82% of initial levels. In contrast, when fibres recovered without glucose, glycogen was still depleted and $[Ca^{2+}]_i$ only recovered to 57% of initial levels. Thus ~25% (82 − 57%) of the recovery of Ca^{2+} release following fatigue appears to be glycogen dependent. It seems reasonable to infer that a similar proportion of the failure of

Ca^{2+} release during fatigue can be attributed to glycogen-dependent mechanisms. A postulated glycogen-dependent mechanism to explain E–C coupling failure during fatigue is outlined in a subsequent section.

Glycogen-independent mechanisms

In the present study there were several conditions where the reductions in $[Ca^{2+}]_i$ and force were not associated with decreases in muscle glycogen concentration. The prolonged reduction of $[Ca^{2+}]_i$ 60 min after fatigue 1 under control conditions as well as the decrease in fatigue resistance following 50 Hz stimulation were associated with the resynthesis of muscle glycogen and appear to be due to glycogen-independent mechanisms. We have recently shown that the prolonged impairment of Ca^{2+} release after a single fatigue bout is due to some Ca^{2+}-activated process (Chin & Allen, 1996). This Ca^{2+}-activated mechanism may have contributed to the reduction in Ca^{2+} release and the reduced fatigue resistance during the second fatigue bout following 50 Hz stimulation. The additional period of high $[Ca^{2+}]_i$ during 50 Hz stimulation may have activated this process. The reduced fatigue resistance after 50 Hz stimulation could also be due to elevations in myoplasmic P_i. It has been shown in skinned muscle fibres that elevations in P_i to levels observed during fatigue (25–50 mM) can decrease both the rate and magnitude of Ca^{2+} release from the SR (Fryer *et al.* 1995). Fryer *et al.* (1995) proposed that elevated levels of myoplasmic P_i may lead to P_i transport into the SR, calcium phosphate precipitation and a reduction in the releasable SR Ca^{2+} pool. Thus, elevated myoplasmic $[Ca^{2+}]_i$ or P_i are two possible glycogen-independent mechanisms that alter force, $[Ca^{2+}]_i$ and fatigue resistance.

Glycogen-dependent mechanisms – a hypothesis for E–C coupling failure in skeletal muscle fatigue

We have shown that, in general, changes in muscle glycogen are associated with changes in fatigue resistance, force and $[Ca^{2+}]_i$ in single muscle fibres. Based on these associations, an important question to consider is how the decrease in muscle glycogen might contribute to E–C coupling failure. As a working hypothesis, we suggest that there is a functional coupling between ATP supplied by glycolysis and ATP utilized within the SR–t-tubule triadic gap (Han, Thieleczek, Varsányi & Heilmeyer, 1992; Xu, Zweier & Becker, 1995). During repeated tetani, when glucose uptake is not rapid enough to supply the energy required for muscle contraction, intracellular PCr and glycogen stores are utilized. When PCr and glycogen are reduced below some critical level, ATP levels may transiently fall before $[Ca^{2+}]_i$ and force output drop. Under these conditions, the net reduction in ATP may inhibit excitation–contraction coupling processes or impair optimal Ca^{2+} release channel function (Smith, Coronado & Meissner, 1985; Allen, Lännergren & Westerblad, 1997). Evidence from intact fibres indicates that the release of caged ATP during fatigue can temporarily reverse the decrease in force and $[Ca^{2+}]_i$ and overcome the impairment in E–C coupling (Allen *et al.* 1997). Although the energy limitation hypothesis of muscle fatigue has been criticized due to the fact that whole-cell ATP concentrations rarely fall by more than 30–50% (Vøllestad *et al.* 1988) and muscle glycogen is never entirely depleted, the concentration of glycogen and ATP within the narrow restricted space of the triadic gap may fall well below that in the bulk space. The compartmentalized supply and utilization of ATP in skeletal muscle triads is supported by observations that: (i) glycogen is bound in distinct regions within mammalian muscle fibres including the lateral ends of the I-band, a region corresponding to the terminal cisternae region of the SR (Fridén *et al.* 1989); (ii) glycogen in the lateral I-band region is preferentially depleted following intense exercise in humans (Fridén *et al.* 1989); and (iii) ATP supplied by glycolytic enzymes is utilized to phosphorylate proteins in the triadic gap that may be required for E–C coupling (Han *et al.* 1992). The role of muscle glycogen may therefore be to provide a rapid and local supply of ATP in the triadic gap for important phosphorylation–dephosphorylation steps (Han *et al.* 1992) or direct binding to the Ca^{2+} release channel (Smith *et al.* 1985). When localized glycogen stores are depleted, ATP supply in the SR triadic region falls and a decrease in Ca^{2+} release and force results. This type of glycogen-dependent mechanism might play an important feedback role in inactivating muscle E–C coupling under conditions of metabolic stress such as during muscle fatigue.

In summary, we have shown that decreases in muscle glycogen concentration contribute to the reduction in force and $[Ca^{2+}]_i$ observed during fatigue. These reductions can be explained by an impairment in Ca^{2+} release from the SR and an inhibition of the myofibrillar proteins. Our observations confirm, at a cellular level, the correlation between muscle glycogen concentration and fatigue and suggest a mechanistic link between muscle glycogen and proteins involved in excitation–contraction coupling and force production.

AHLBORG, B., BERGSTRÖM, J., EKELUND, L.-G. & HULTMAN, E. (1967). Muscle glycogen and muscle electrolytes during prolonged physical activity. *Acta Physiologica Scandinavica* **70**, 129–142.

ALLEN, D. G., DUTY, S. & WESTERBLAD, H. (1993). Metabolic changes in muscle during exercise; their effects on muscle function. *Proceedings of the Australian Physiological and Pharmacological Society* **24**, 65–75.

ALLEN, D. G., LÄNNERGREN, J. & WESTERBLAD, H. (1995). Muscle cell function during prolonged activity: cellular mechanisms of fatigue. *Experimental Physiology* **80**, 497–527.

ALLEN, D. G., LÄNNERGREN, J. & WESTERBLAD, H. (1997). Role of ATP in the regulation of Ca^{2+} release in normal and fatigued mouse skeletal muscle. *Journal of Physiology* (in the Press).

ALLEN, D. G., LEE, J. A. & WESTERBLAD, H. (1989). Intracellular calcium and tension during fatigue in isolated single muscle fibres from *Xenopus laevis*. *Journal of Physiology* **415**, 433–458.

BELCASTRO, A. N., ROSSITER, M., LOW, M. P. & SOPPER, M. M. (1981). Calcium activation of the sarcoplasmic reticulum ATPase following strenuous activity. *Canadian Journal of Physiology and Pharmacology* **59**, 1214–1218.

Bergström, J., Hermansen, L., Hultman, E. & Saltin, B. (1967). Diet, muscle glycogen and physical performance. *Acta Physiologica Scandinavica* **71**, 140–150.

Bonen, A. & Homonko, D. A. (1994). Effects of exercise and glycogen depletion on glyconeogenesis in muscle. *Journal of Applied Physiology* **76**, 1753–1758.

Byrd, S. K., Bode, A. K. & Klug, G. A. (1989). Effects of exercise of varying duration on sarcoplasmic reticulum function. *Journal of Applied Physiology* **66**, 1383–1389.

Chin, E. R. & Allen, D. G. (1996). The role of elevations in intracellular $[Ca^{2+}]$ in the development of low frequency fatigue in mouse single muscle fibres. *Journal of Physiology* **491**, 813–824.

Cooke, R., Franks, G., Luciano, G. & Pate, E. (1988). The inhibition of rat skeletal muscle contraction by hydrogen ions and phosphate. *Journal of Physiology* **395**, 77–97.

Douen, A. G., Ramlal, T., Rastogi, S., Bilan, P. J., Cartee, G. D., Vranic, M., Holloszy, J. O. & Klip, A. (1990). Exercise induces recruitment of the 'insulin responsive glucose transporter'. Evidence for distinct intracellular insulin- and exercise-recruitable transporter pools in skeletal muscles. *Journal of Biological Chemistry* **265**, 13427–13430.

Entman, M. L., Keslensky, S. S., Chu, A. & van Winkle, W. B. (1980). The sarcoplasmic reticulum–glycogenolytic complex in mammalian fast twitch skeletal muscle. Proposed *in vitro* counterpart of the contraction-activated glycogenolytic pool. *Journal of Biological Chemistry* **255**, 6245–6252.

Favero, T. G., Pessah, I. N. & Klug, G. A. (1993). Prolonged exercise reduces Ca^{2+} release in rat skeletal muscle sarcoplasmic reticulum. *Pflügers Archiv* **422**, 472–475.

Fitts, R. H. (1994). Cellular mechanisms of muscle fatigue (Review). *Physiological Reviews* **74**, 49–94.

Fridén, J., Seger, J. & Ekblom, B. (1989). Topographical localization of muscle glycogen: An ultrahistochemical study in human vastus lateralis. *Acta Physiologica Scandinavica* **135**, 381–391.

Fryer, M. W., Owen, V. J., Lamb, G. D. & Stephenson, D. G. (1995). Effects of creatine phosphate and P_i on Ca^{2+} movements and tension development in rat skinned skeletal muscle fibres. *Journal of Physiology* **482**, 123–140.

Galbo, H., Holst, J. J. & Christensen, H. J. (1979). The effect of different diets and of insulin on the hormonal response to prolonged exercise. *Acta Physiologica Scandinavica* **107**, 19–32.

Godt, R. E. & Nosek, T. M. (1989). Changes in the intracellular milieu with fatigue or hypoxia depress contraction of skinned rabbit skeletal and cardiac muscle. *Journal of Physiology* **412**, 155–180.

Han, J., Thieleczek, R., Varsányi, M. & Heilmeyer, L. M. G. (1992). Compartmentalised ATP synthesis in skeletal muscle triads. *Biochemistry* **31**, 377–384.

Lännergren, J. & Westerblad, H. (1991). Force decline due to fatigue and intracellular acidification in isolated fibers from mouse skeletal muscle. *Journal of Physiology* **434**, 307–322.

Lee, J. A., Westerblad, H. & Allen, D. G. (1991). Changes in tetanic and resting $[Ca^{2+}]_i$ during fatigue and recovery of single muscle fibres from *Xenopus laevis*. *Journal of Physiology* **433**, 307–326.

Lindinger, M. I., Heigenhauser, G. J. F. & Spriet, L. L. (1987). Effects of intense swimming and tetanic electrical stimulation on skeletal muscle ions and metabolites. *Journal of Applied Physiology* **63**, 2331–2339.

Lowry, O. H. & Passonneau, J. V. (1972). *A Flexible System of Enzymatic Analysis*. Academic Press, New York.

Nagesser, A. S., van der Laarse, W. J. & Elzinga, G. (1992). Metabolic changes with fatigue in different types of single muscle fibres of *Xenopus laevis*. *Journal of Physiology* **448**, 511–523.

Smith, J. S., Coronado, R. & Meissner, G. (1985). Sarcoplasmic reticulum contains adenine nucleotide-activated calcium channels. *Nature* **316**, 446–449.

Spriet, L. L. (1992). Anaerobic metabolism in human skeletal muscle during short-term, intense activity. *Canadian Journal of Physiology and Pharmacology* **70**, 157–165.

Spriet, L. L., Heigenhauser, G. J. F. & Jones, N. L. (1986). Endogenous triacylglycerol utilization by rat skeletal muscle during tetanic stimulation. *Journal of Applied Physiology* **60**, 410–415.

Vøllestad, N. K., Sejersted, O. M., Bahr, R., Woods, J. J. & Bigland-Ritchie, B. (1988). Motor drive and metabolic responses during repeated submaximal contractions in humans. *Journal of Applied Physiology* **64**, 1421–1427.

Westerblad, H. & Allen, D. G. (1991). Changes in myoplasmic calcium concentration during fatigue in single mouse muscle fibres. *Journal of General Physiology* **98**, 615–635.

Westerblad, H. & Allen, D. G. (1992). Changes of intracellular pH due to repetitive stimulation of single fibres from mouse skeletal muscle. *Journal of Physiology* **449**, 49–71.

Westerblad, H. & Allen, D. G. (1993). The influence of intracellular pH on contraction, relaxation and $[Ca^{2+}]_i$ in intact single fibres from mouse muscle. *Journal of Physiology* **466**, 611–628.

Westerblad, H. & Allen, D. G. (1994). The role of sarcoplasmic reticulum in relaxation of mouse muscle; effects of 2,5-di(*tert*-butyl)-1,4-benzohydroquinone. *Journal of Physiology* **474**, 291–301.

Westerblad, H., Duty, S. & Allen, D. G. (1993). Intracellular calcium concentration during low-frequency fatigue in isolated single fibers of mouse skeletal muscle. *Journal of Applied Physiology* **75**, 382–388.

Xu, K. Y., Zweier, J. L. & Becker, L. C. (1995). Functional coupling between glycolysis and sarcoplasmic reticulum Ca^{2+} transport. *Circulation Research* **77**, 88–97.

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